Immunofluorescence Localization of Glycoproteins Using Tissue Printing: Detection of Pistil Extensin-Like Proteins in Tobacco

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Immunocytochemical methods often encounter difficulties with both soluble antigens and with processing large numbers of samples efficiently. As a complement to in situ hybridization (5), western blotting and ultrastructural immunolocalization (3), we searched for a suitable method to gain information on the overall subcellular localization of highly soluble extensin-like glycoproteins in Nicotiana tabacum (tobacco) pistils at various stages of development. Whereas the cryofixation technique that is used in conjunction with embedding, sectioning and immunofluorescence labeling (1) limits the risks of translocation or loss of epitopes during the preparation procedure, this technique is time-consuming and not appropriate for autofluorescent organs like tobacco pistils. The tissue print-alkaline phosphatase (AP) detection method (see References 2, 10, 13 and 14 for general references 2, 10, 13 and 14 for general


tions of Pistil Extensin-Like Proteins in Tobacco

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Benchmarks

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ric acid and 44 mM Na$_2$HPO$_4$. After 20 min, the reaction was stopped using 200 mM citric acid. The color reaction was analyzed by direct vision and by use of a Dialux® 20EB Transmission Microscope (Leitz, Wetzlar, Germany) with a strong light source and low magnification.

Additionally, the distribution of the pistil extensin-like glycoproteins was investigated in pollinated mature styles. Strings of transmitting tissue were dissected from these styles and firmly adhered to pieces of nitrocellulose that functioned as a physical support during the labeling procedure, which was carried out as described above. These samples were first examined for FITC immunolabeling and then post-stained with the UV-excitation, blue-emission dye Aniline blue (Polysciences, War- rington, PA, USA), which is an ideal marker for callose plugs that are typical of pollen tubes (8).

Routine examination of preparations was carried out with an Orthoplan® Fluorescence Microscope (Leitz) equipped with Leitz fluoratar objectives, a HBO 200W High-Pressure Mercury Lamp (Osram, Berlin, Munich, Germany) and fluorescence filter sets compatible with FITC visualization that consisted of a 450–490-nm excitation filter, a 510-nm dichroic mirror and a 515-nm barrier filter. Image documentation was obtained with a Vario Ortomat® Photographic Unit (Leitz) using Kodak® 200 ASA Ektachrome Color Slide Films. In addi- tion, digital images were made with an MRC 600® Confocal Laser Scanning Microscope (CLSM; Bio-Rad, Hemel Hempstead, England, UK), mounted on an inverted Diaphot® Microscope (Ni- kon, Tokyo, Japan). The 488-nm excitation line of the 20-mW argon laser was set at 30% transmission intensity. The BHS filter set of the MRC 600 consisted of a 488DF10 excitation filter, a 510-nm dichroic mirror and a 515-nm barrier filter.

With the present immunofluorescence method, we were able to analyze about 500 samples within two months (at least 15 tissue prints per stage using the antibody and 10 tissue prints per stage for each type of control) and make detailed observations at the tissue and cellular levels (for other examples of fluorescent signal detection on prints without imaging of structures, see Reference 12). Tissue prints of tobacco pistils treated with both the primary and secondary antibody showed an intense apple-green fluorescence covering the area corresponding to the entire transmitting track from stage 4 on (not earlier; Figure 1A). There was no antiglyco- protein-related labeling in the other tissues of the pistil. At low magnification (4×, 10× or 25× objectives), the specific labeling appeared as a distinct and sharp band in young styles (from stage 4 to 8; 14 or 15 positives out of 15 tissue prints per stage, Reference 4; Figure 1A), and as a bright uniform mark with a more fluid appearance over the whole length of the transmitting track in mature unpollinated styles (stage 11 + 0, 24 or 48 h; all positive out of, respectively, 15, 25 and 16 tissue prints; see extensive data in Reference 3). Combined prints showed similar levels of signal 24 h after pollination, but the labeling intensity declined markedly 48 h after pollination (3 weakly positive out of 27 tissue prints). The antiglycoprotein signal observed in the area concurring to the transmitting track clearly contrasted in color, intensity and aspect with the background fluorescence. This background light had multiple origins. First, the fibers in the nitrocellulose membrane provided a faint yellowish glow, which was also present in unprinted membranes that were incubated only in buffer or secondary antibody (not shown). Second, unprinted membranes were weakly and uniformly stained after contact with rabbit serum and the secondary antibody (see unprinted area above the edge of the material print in Figure 1B). This problem could not be reduced by pre-blocking with 20% fetal calf or goat sera. Third, both preimmune and immune sera collected from three rabbits appeared to have a weak nonspecific
affinity for unidentified products of the pistil that were visible as faint yellowish spots and/or as a weak shine over the entire tissue printed area. This signal could be fully eliminated by incubating the prints with 20% goat serum for 1 h, before the incubation with either preimmune serum, primary antibody or buffer. In addition, some red autofluorescence, caused by remnants of tissue or by wounding products was also visible in some samples. This autofluorescence signal together with the yellowish nonspecific spots seen in unblocked tissue prints, provided a useful mark to interpret the locations of different tissues in the prints. Importantly, the distinction between specific and nonspecific fluorescence was very consistent (compare Figure 1, A and B; all 222 control tissue prints were negative). At higher magnifications (40× or 63× and 100× oil immersion objectives), labeled prints of single transmitting track cells were clearly visible (Figure 1C). This pattern was consistent with the fluorescence labeling observed at the surface of transmitting track cells adhered to the nitrocellulose membranes (Figure 1C). The tissue print data were also in agreement with the results of the immunogold labeling experiments showing labeling in the intercellular space at the ultrastructural level (3). However, comparison of results from tissue printing vs. observations on transmitting tissue adhered to membranes indicated that epitopes located within intact transmitting tissue and in pollen tubes (not shown) remained undetected in tissue prints, probably because of poor accessibility of these compartments to large antibodies. Although fluorescent patterns were sharply visualized with the CLSM, the filter combination used was not selective enough to discriminate the FITC signal specific for pistil extensin-like proteins from auto- and background-fluorescence signals (Figure 1D).

The specificity and image quality of tissue prints in which a peroxidase reaction had been used for detection was very poor (results not shown). Although remnants of transmitting track cells were intensely stained after incubation with the primary and HRP secondary antibodies, several problems were found. The nitrocellulose membrane showed an overall blue color reaction, except for the clear demarcation corresponding to the area of the stigma in the tissue print. However, dark-blue spots were visible at the level of the upper papillary rim. These spots were also present in tissue prints that had not been exposed to the primary serum, suggesting that the reaction was caused by endogenous enzymes. Next, when either preim-

Figure 1. Immunofluorescence localization of pistil extensin-like glycoproteins (A, C, D) and pre-immune control (B) on tissue prints of tobacco pistils. (A–C) Conventional fluorescence microscopy in unprocessed images and (D) CLSM. (A) Overview of the anti-glycoprotein labeling in a tissue print of a young pistil (total length of the flower bud: 17 mm, stage 4 according to Reference 4). The glycoprotein-specific FITC fluorescence is confined to the transmitting track (arrows), which is located in the center of the style. The stigma (sg) and the cortical area (co) of the style are not labeled. (B) Absence of labeling in a tissue print of a young pistil (stage 4, as in Panel A) that was incubated in preimmune serum and secondary antibody. Only weak, yellowish background (bg) fluorescence and red autofluorescence (au) are apparent in this print, which is representative of the average background pattern. (C) Detail of the anti-glycoprotein labeling in a tissue print of an unpollinated mature pistil (length of the flower is 46 mm, stage 11 according to Reference 4). The labeling is visible both on prints left by single transmitting track cells (arrows) and on the surface of remnants of such cells (open pointers). Red autofluorescence signal, probably caused by chlorophyll, is also apparent inside the cytoplasm of transmitting track cells that adhered. The cortical area (co) of the style is not labeled. (D) CLSM image of the fluorescence pattern in a tissue print of an unpollinated mature pistil (stage 11 according to Reference 4). Glycoprotein-specific labeling is present at the level of transmitting track (arrows; here about 8 mm under the stigma). However, this signal shows a similar fluorescence intensity as the nonspecific background (bg) and autofluorescent (au) remnants of transmitting tissue. Glycoprotein-specific and nonspecific signal can only be differentiated on account of the structure of the patterns, after observation of the true colors in the same field using a conventional fluorescence microscope. Scale bars follow: for Panels A and B, as shown in Panel A, 200 µm; Panel C, 20 µm; Panel D, 100 µm.
mune- or immune-rabbit serum had been used for the first incubation, the entire region covered by the style showed brownish spots. This staining could not be prevented by preincubation of the tissue prints with 20% goat serum.

In conclusion, combining tissue printing and indirect epifluorescence immunolabeling is an easy, rapid and reliable method that is suitable for localizing soluble proteins at tissue and subcellular levels in large samples. With this method, we obtained consistent results that helped establish the developmental expression pattern of the mobile fraction of a new class of soluble glycopeptides in tobacco and related species (See Reference 3 for further data). The results that helped establish the developmental expression pattern of the mobile fraction of a new class of soluble glycopeptides in tobacco and related species could not be prevented by preincubation of the tissue prints with 20% goat serum.

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